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Acetaldehyde-protein adducts, but not lactate and pyruvate, stimulate gene transcription of collagen and fibronectin in hepatic fat-storing cells

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Hepatic fibrosis is an important morphological feature of alcohol-induced liver injury. We previously reported that acetaldehyde, but not ethanol can stimulate type I collagen and fibronectin synthesis in cultures of rat fat-storing cells (FSC) by increasing transcription of the specific genes. The effect of lactate and pyruvate was studied on collagen I, III, fibronectin accumulation by cultured rat FSCs and it was investigated whether acetaldehyde could increase procollagen I and fibronectin gene transcription through the formation of protein adducts. Lactate and pyruvate (5, 15 and 25 mmol/l) did not significantly affect collagen I, III and fibronectin production by cultured FSCs. Pyridoxalphosphate and *p*-hydroxymecuribenzoate (inhibitors of acetaldehyde-protein adduct formation) blocked the stimulatory effect of acetaldehyde on procollagen I and fibronectin gene transcription. These data suggest that ethanol may act as a liver fibrogenic factor through acetaldehyde, its immediate metabolite, whereas lactate does not seem to play a role. Acetaldehyde might stimulate gene transcription of extracellular matrix components by liver FSCs through the formation of adducts with proteins.

Key words: Fat-storing cell; Collagen; Fibronectin; Acetaldehyde

Collagen accumulation in the space of Disse and in the interstitium around hepatocytes is an important morphological feature of alcohol-induced liver injury. However, the mechanisms responsible for increased collagen deposition are still unclear.

Fat-storing cells (FSCs), also known as hepatic lipocytes or Ito cells, reside in the space of Disse and are generally believed to be a major source of collagen and other extracellular matrix components both in vivo and in vitro (1–10). We recently reported that acetaldehyde (AcAld) stimulates collagen (Coll) type I production in cultured rat FSCs but not in hepatocytes, whereas Coll type III and laminin were not affected; in the same experimental model ethanol had no effect on either FSC or hepatocyte cultures (5). More recently, AcAld was shown to increase procollagen type I and fibronectin (FN) gene transcription in rat FSCs through a protein synthesis-dependent mechanism. It was also hypothesized that the AcAld-mediated effect was due to the formation of AcAld-protein adducts (11).

Conflicting results have been recently reported on the effect of lactate and pyruvate on collagen accumulation in different experimental models. Cerbon-Ambriz and coworkers reported that lactate and pyruvate increased collagen production in liver slices and FSCs from CCl_4-treated rats (12,13). In another study, however, lactate was not shown to affect procollagen I and FN mRNAs in cultured rat FSCs (11). Therefore, the role of lactate and pyruvate on the liver collagen accumulation is still under debate.

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In this study we verified whether the stimulatory effect of AcAld on Coll I and FN could be a result of the formation of AcAld-protein adducts. Moreover, the effect of lactate and pyruvate was studied on Coll I, III and FN production by rat FSCs in order to clarify whether lactate might affect collagen and FN synthesis at a posttranscriptional level as it failed to enhance the specific mRNA expression (11).

Materials and Methods

Materials

Iscove's modified Dulbecco's medium (D-MEM), heat-inactivated fetal calf serum (FCS), L-glutamine, Hanks' balanced salt solution (HBSS), penicillin/streptomycin, fungizone and sodium bicarbonate were purchased from Grand Island Biological, Grand Island, NY. Collagen type I (rat tail), collagenase type I, protease type XIV (Pronase E), lactate, pyruvate, pyridoxal-5′-phosphate, *p*-hydroxymercuribenzoate, beta-aminoproprionitrile (β-APN), monoclonal anti-desmin antibody (clone DE-U-10), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG $F(ab')_2$, chondroitin sulfate A and cetylpyridinium chloride (CPC) were obtained from Sigma Chemical Co., St. Louis, MO. Nycodenz was obtained from Accura Chemical & Scientific, Westbury, NY. Collagen type III (human placenta), FN (bovine plasma) and rabbit anti-FN polyclonal antibodies were purchased from Calbiochem, La Jolla, CA. Rabbit anti-monkey type I and anti-rat type III polyclonal antibodies were kindly supplied by Dr. D. Schuppan, Department of Gastroenterology, Klinikum Steglitz, Free University of Berlin, Germany. Tissue culture plastic dishes were obtained from Falcon Div., Becton Dickinson, Oxnard, CA. Agarose was obtained from FMC Bio Products, Rockland, ME. Phenol was obtained from IBI, New Haven, CT. GeneScreens were obtained from DuPont NEN, Wilmington, DE. L-[2,3,4,5-^{3}H]proline (specific activity, 100 Ci/mmol) was obtained from ICN Radiochemicals, Irvine, CA. [^{32}P]dCTP and [α-^{32}P]UTP (specific activity, 3 000 Ci/mmol and >400 Ci/mmol, respectively), and [1,2-^{14}C]AcAld (5 mCi/mmol) were obtained from NEN, Berlin, Germany. Glass fiber filters GF/F were obtained from Whatman Biochemicals, Maidstone, Kent, UK. Acetaldehyde was obtained from Eastman Kodak Co., Rochester, NY.

Preparation of fat-storing cells

FSCs were isolated from old female Sprague–Dawley rats (500–700 g body weight) that had free access to water and a standard chow diet. Non-parenchymal liver cells were isolated by the pronase-collagenase method of Knook et al. (14) with some minor modifications (5). Briefly, after a 10-min perfusion with Ca^{2+}/Mg^{2+}-free HBSS (pH, 7.4; flow rate, 10 ml/min) the liver was digested with HBSS containing 0.1% pronase E and 0.05% collagenase type I for a further 10 min at the same flow rate. The liver was then removed and incubated in HBSS containing 0.05% collagenase and 0.02% pronase at 37°C for 20 min. FSCs were obtained by centrifugation over a 11.4% (w/v) Nycodenz gradient (9) for 17 min at 1400 × *g*. After centrifugation, FSCs were resuspended in Iscove's D-MEM containing 10% FCS, 2 mmol/l L-glutamine and antibiotics. Viability of the cells was >90%, as evaluated by the trypan blue exclusion test. Cells were then seeded on 100-mm plastic tissue culture dishes at a starting density of 1×10^6 cells/10 cm^2 and incubated at 37°C in a 5% CO_2-air humified atmosphere. Contaminating sinusoidal endothelial and Kupffer cells were checked in the primary cultures by factor VII-associated antigen (15) immunofluorescence (IF) and endogenous peroxidase activity, respectively. FSCs were identified by the phase-contrast light microscopic appearance, the vitamin A autofluorescence and the constant positive IF staining for desmin even after the first and second passage in vitro (16). Desmin staining in primary cultures was performed 8 days after seeding to avoid low desmin expression previously described in freshly isolated or early primary cultures (17).

Indirect immunofluorescence

Small aliquots of cells were cultured for indirect IF studies on Lab-Tek chamber slides and processed as previously described (5). Briefly, slides were incubated with monoclonal anti-desmin antibody (1:10 diluted in PBS) for 30 min at room temperature in a humified chamber, then washed 3 × 5 min in PBS and incubated for another 30 min at room temperature with FITC-conjugated anti-mouse IgG-$F(ab')_2$, diluted 1:20 in PBS containing 10% normal rat serum. Controls were performed by replacing the first antibody with a mouse total IgG_1, at the same dilution as the monoclonal antibody. Slides were examined with a Zeiss fluorescence microscope equipped with epiillumination.

Cell incubations with acetaldehyde, lactate or pyruvate

Airtight 100-mm culture dishes were used for AcAld incubations. FSCs (passage 1 and 2 cultures) were incubated with 175 μmol/l AcAld (initial concentration) in serum-free medium containing 50 μg/ml ascorbic acid and 100 μg/l β-APN for 6 or 24 h at 37°C. Some incuba-

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tions with AcAld were carried out with 10 mmol/l pyridoxal-5′-phosphate (PLP) or 4 μmol/l *p*-hydroxymercuribenzoate (PHMB), two inhibitors of AcAld-protein adduct formation (18). PHMB was added to the cells in serum-free medium without AcAld for 1 h at 37°C; FSCs were then washed 3 times with HBSS to remove the excess PHMB (18) and exposed to AcAld for 24 h as described above. PLP and AcAld were added to the cultures together (18). The concentrations of AcAld was monitored by a head space gas chromatograph model F 42 (Perkin Elmer, Norwalk, CT) (5). For experiments with lactate and pyruvate, cells were cultured in the same 100-mm plastic dishes at 37°C for 24 h with 3 different concentrations (5, 15 and 25 mmol/l) of lactate and pyruvate in serum-free medium.

AcAld-protein adduct determination in the cultures

[1,2-^{14}C]AcAld (5 mCi/mmol) was added to FSC cultured on airtight 35-mm Petri dishes for 24 h in serum-free medium at a starting concentration of 175 μmol/l (875 nCi/1 ml of medium). Media were then removed and collected. Cells were harvested in 5% trichloroacetic acid (TCA). Media and cell monolayers were separately processed for protein precipitation with 5% TCA on ice for 30 min. After centrifugation at 2000 × *g* for 15 min, pellets were washed 3 times with ethanol/ether (3/1, v/v) at 4°C and resuspended in 5% sodium dodecyl sulfate (SDS) (w/v in water) by vortexing. Radioactivity was determined in a beta-counter after addition of 10 ml of scintillation liquid.

Collagen assay

Collagen type I, III and fibronectin were determined in culture media and cell layers by an enzyme-linked immunoassay (ELISA) method as previously described (5). Standards and samples were assayed in triplicate. The detection limits for collagen type I, III and FN were 2, 2.5 and 2.5 ng/well, respectively. There was no detectable cross-reactivity between different collagen types. Results were expressed as ng collagen/μg cellular DNA.

RNA extraction

RNA was extracted from FSCs as described elsewhere (11) with some minor modifications. Briefly, cells were washed twice in phospate buffer solution (PBS), pH 7.4, scraped with a rubber policeman and pellets made by centrifugation at 450 × *g* for 10 min. Total RNA was then isolated by the method of Chomczynski and Sacchi (19), precipitated with isopropanol, washed twice with 75% ethanol and resuspended in DEP water. Total RNA content of the samples was calculated by A260 spectrophotometry.

Northern blot hybridization

Steady-state mRNA levels were measured by Northern blot hybridization according to standard techniques (20). Ten micrograms of denatured total RNA per sample were electrophoresed in a 1% agarose-3% formaldehyde gel, transferred to a GeneScreen filter, which was then baked for 2 h at 80°C in a vacuum oven. Equal loading of the samples was checked by ethidium bromide staining on a ultraviolet light box and by reprobing the blots with α-tubulin cDNA after the hybridization procedures. Filters were prehybridized and then hybridized overnight at 42°C with the following cDNA probes: rat pro α1(I) collagen (21) and rat total fibronectin (FN) (22). cDNA probes were labeled with [^{32}P]dCTP to a specific activity of 2–5 × 10^8 counts/min/μg DNA, by using a random-primer kit (Amersham). After hybridization, filters were washed to a maximum stringency of 0.1× SSC/0.1% SDS at 65°C. Filters were then exposed to Kodak XAR-5 film at −70°C, developed and quantitated by scanning laser densitometry.

Run-on transcription

The nuclear run-on transcription assay was carried out as previously described (11). Briefly, 6 × 10^7 cells/sample were scraped and then lysed by adding 10% Triton X-100, followed by Dounce homogenization. The nuclei were pelleted by centrifugation at 500 × *g* for 10 min. The nuclear transcription reaction was immediately run at 30°C for 20 min as reported elsewhere (11). The [α-^{32}P]UTP-radiolabeled RNA was purified according to the method of Chomczynski and Sacchi (19). Linearized plasmid DNAs, and the plasmid alone as a negative control (5 μg/slot), were applied to nitrocellulose paper using a slot blotter. Nitrocellulose filters were prehybridized at 42°C for 4 h, hybridized with the radiolabeled RNA at 42°C for a further 48 h and then washed to a maximum stringency of 0.1× SSC/0.1% SDS at 65°C. Filters were then exposed to X-ray film at −70°C and the autoradiograms quantitated by scanning laser densitometry.

Total protein synthesis

The incorporation of tritiated proline into protein was used to measure total protein synthesis in the cell cultures as previously described (5). Briefly, L-[2,3,4,5-^{3}H]proline (1 μCi/ml; final concentration [^{3}H]proline, 0.01 μmol/l; final concentration unlabelled proline, 0.25 mmol/l) was added to FSC cultures during the incubation period. Media and cells were then harvested separately and stored at −20°C. The total incorporation of [^{3}H]proline into protein was determined by TCA precipitation (23).

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The free intracellular proline pool, determined in the cultures by a gas chromatographic-mass spectometry procedure (24), was modified by acetaldehyde, as previously reported (5).

Statistics

Data were expressed as mean ± 1 standard deviation (S.D.). Statistical analysis was performed by ANOVA (5).

Results

The proportion of cells staining positive for desmin was 93–95% in primary FSC culture and 99% after the first and second passages, as previously reported (5). A lower desmin positivity (about 50%) has been found in freshly isolated or early (3 day) primary cultures of FSCs (17). However, the same authors reported that most of the cells were desmin-positive after 7 days of primary culture (desmin was checked for at day 8). The decrease in AcAld concentration in FSC cultures was time-dependent. The rate of AcAld disappearance from media was higher in the presence of cells than in media without cells (Fig. 1). Concentrations at 24 h were 42 ± 9 μmol/l with and 84 ± 5 without cells ($P < 0.005$).

AcAld increased Coll I and FN production 2.5- and 2.3-fold in FSC cultures, respectively, whereas Coll III accumulation was not significantly modified. Lactate and pyruvate (5, 15, 25 mmol/l) did not affect Coll I, III and FN production (Table 1).

TABLE 1
Collagen and fibronectin production in FSC cultures

	Coll I (ng/μg DNA)	Coll III (ng/μg DNA)	FN (ng/μg DNA)
Control	920 ± 107	380 ± 54	178 ± 32
Acetaldehyde	2308 ± 315*	396 ± 72	412 ± 61*
Lactate			
5 mM	830 ± 24	308 ± 30	208 ± 54
15 mM	922 ± 100	348 ± 45	184 ± 28
25 mM	756 ± 30	310 ± 41	175 ± 18
Pyruvate			
5 mM	826 ± 97	362 ± 32	191 ± 27
15 mM	845 ± 74	393 ± 96	172 ± 31
25 mM	894 ± 81	338 ± 43	187 ± 22

Data are mean values ± S.D. of 5 experiments perfomed in triplicate. Passage 1 and 2 early confluent cultures showing the 'activated' phenotype of FSC (myofibroblast-like) were used. (mM = mmol/l.)
*$P < 0.001$ vs. control.

PLP or PHMB (inhibitors of AcAld-protein adduct formation) blocked the stimulatory effect of AcAld on Coll I and FN synthesis in FSC cultures (Fig. 2), without affecting total protein synthesis (97 ± 10% and 104 ± 8% of controls, respectively; controls = 2175 ± 227 dpm/μg cellular DNA). When cells were pretreated with PHMB, the amount of TCA-precipitable [^{14}C]-AcAld was largely reduced after 24 h of incubation compared with FSCs incubated with AcAld alone. This

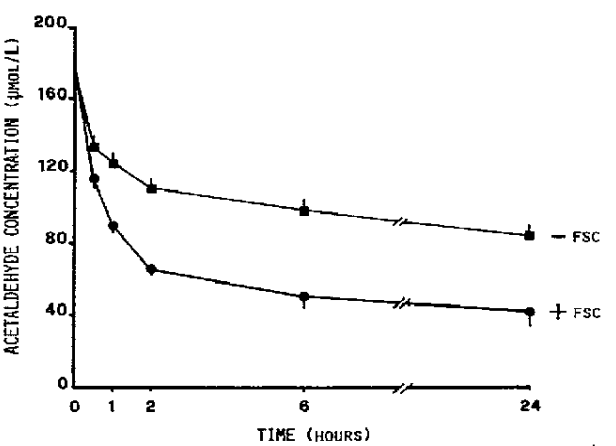

Fig. 1. Concentrations of AcAld in the cell culture media during the 24 h period of incubation. The rate of AcAld disappearance was higher in the presence of cells (+FSC) than in media without cells (–FSC). Data shown are mean values ± S.D. (S.D. = vertical bars) of 5 experiments in triplicate. ($P < 0.005$ at $t = 24$ h.)

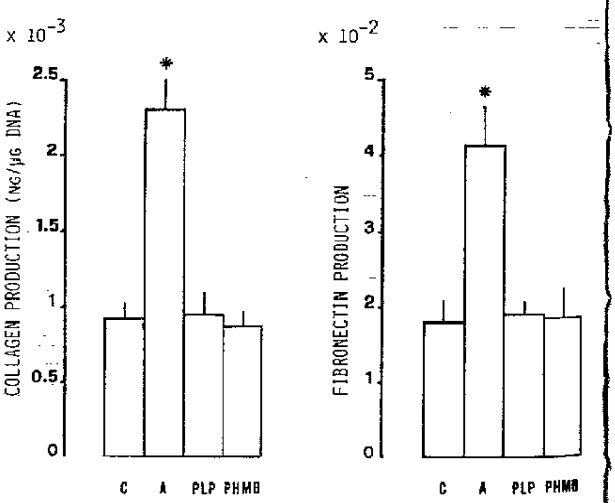

Fig. 2. Pyridoxal-phosphate (PLP; 10 mmol/l) and *p*-hydroxymercuribenzoate (PHMB; 4 μmol/l) completely blocked the stimulatory effect of acetaldehyde (A) on collagen type I (left panel) and fibronectin (right panel) accumulation by cultured rat FSCs. Data (ng/μg of cellular DNA) are mean ± S.D. of 5 experiments performed in triplicate. C = controls. *$P < 0.001$.

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TABLE 2
Effect of PHMB and PLP on the TCA-precibitable [^{14}C]AcAld in fat-storing cell layer and media

	AcAld (dpm/μg DNA)	AcAld + PHMB (dpm/μg DNA)	AcAld + PLP (dpm/μg DNA)
Cells	8789 ± 964	3161 ± 245*	1148 ± 230*
Media	158 ± 32	127 ± 29	145 ± 24

Data are mean values ± S.D. of 5 experiments performed in triplicate.
*P < 0.001 vs. AcAld alone in the cells.

effect was particularly evident with PLP. Insignificant amounts of radioactivity were recovered in the culture media after TCA precipitation (Table 2). The trypan blue exclusion test did not show any toxic effects for the two compounds on FSCs at the end of the 24-h incubation period.

Northern blot analysis demonstrated that the inhibitory effect of these compounds occurred at the level of gene expression (Fig. 3). Run-on assay confirmed previous findings (11) of an AcAld stimulatory effect of Coll I and FN gene transcription and showed a total inhibition of this effect by PLP (both on Coll I and FN)

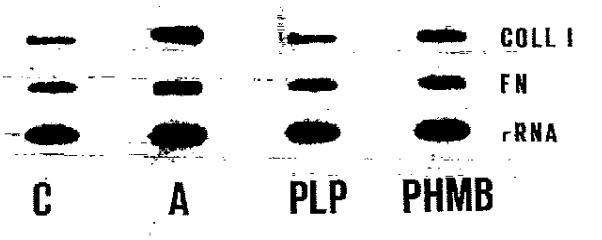

Fig. 4. Effect of PLP and PHMB on the acetaldehyde-induced increase of procollagen I (COLL I) and fibronectin (FN) gene transcription in cultured FSCs. Cells were cultured in the absence (C) or in the presence (A) of acetaldehyde for 3 h; nuclei were then isolated and a run-on assay was performed. The acetaldehyde-induced (A) increase of COLL I and FN gene transcription was 3.5- and 2.5-fold, respectively, by scanning laser densitometry. Densitometry data were corrected by variations of the 28S ribosomal (r) RNA, used as a control. PLP completely blocked this stimulation of COLL I and FN gene transcription. PHMB partially inhibited the enhancement of COLL I gene transcription induced by acetaldehyde (2-fold, by laser densitomery). The plasmid alone (pGEM), used as a negative control, did not show any hybridization signal.

and a partial inhibitory effect by PHMB (Fig. 4). The AcAld-induced increase of Coll I and FN gene transcription was 3.5- and 2.5-fold, respectively, by scanning laser densitometry (Fig. 4).

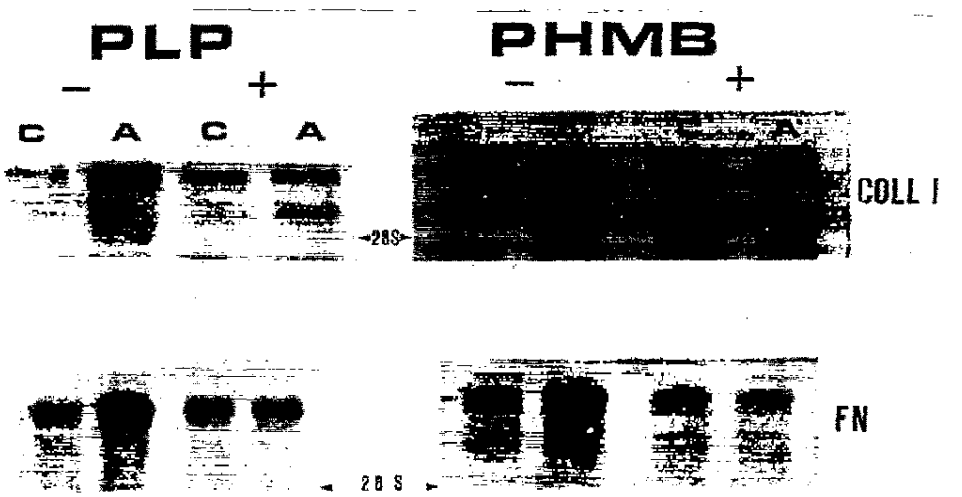

Fig. 3 Expression of α1(I) procollagen (COLL I) and fibronectin (FN) mRNAs in rat FSCs cultured with (A) or without (C) acetaldehyde for 6 h in the absence (−) or in the presence (+) of pyridoxal-phosphate (PLP) and *p*-hydroxymercuribenzoate (PHMB). Two transcripts were present for COLL I (5.8 and 4.8 kb). The FN transcript was 9.1 kb. Note that both PLP and PHMB completely blocked the stimulatory effect of acetaldehyde.

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Discussion

In this study neither lactate nor pyruvate were found to significantly modify the production of Coll I, III and FN by cultured FSCs. These results are in agreement with a previous report (11). Other studies on the stimulatory effect of lactate on collagen production (12,13,25) used much higher lactate concentrations (30 mmol/l) than those found in the blood of alcoholics (less than 5 mmol/l) (26). Although lactate concentrations may be higher in the liver than in the blood after alcohol consumption, lactate did not affect collagen and FN production by cultured FSCs even at 25 mmol/l (more than 5-fold the peak blood levels). Actually, the only report that does not support the findings of this study is the study by Cerbon-Ambriz et al. (13), which demonstrates the stimulatory effect of lactate on total collagen production by rat FSCs. In fact, some studies were performed in different cell types (baboon liver myofibroblasts in the study by Savolainen et al. (25) and rat FSCs here) and others were carried out using rat liver slices, making it impossible to identify which cell type was involved in collagen production (12). On the other hand, Cerbon-Ambriz et al. (13) used FSCs isolated from CCl_4-treated rats and measured total collagen production by proline incorporation. Evaluation of the different collagen types and extracellular matrix components is not possible with that method. Observations in this study are based on the use of FSCs from normal rats and the use of an ELISA procedure with specific antibodies for Coll I, III and FN.

FSCs cultured on plastic have been shown to change their phenotype with a decrease in fat droplets and an increase in rough endoplasmic reticulum within 6 days in primary culture. This 'differentiation' parallels a marked increase in collagen production (27). Thus, FSCs transform into myofibroblast-like cells within the first week of primary culture and probably maintain this 'intermediate' phenotype up to the first two passages on plastic as demonstrated by constant desmin positivity (16). Passage 1 and 2 FSC cultures were used because early passaged cells produce collagen amounts similar to those found in confluent primary cultures (5). When liver injury occurs (alcohol-induced, for example) FSCs turn into 'transitional' cells (28) with morphological characteristics similar to FSCs cultured on plastic. Therefore, the in vitro model in this study mimics lipocyte activation in vivo and increased collagen production after alcohol feeding.

AcAld, but not ethanol, has been reported to stimulate collagen production in different, mostly 'non-liver specific', experimental models (25,29,30). AcAld was recently shown to increase procollagen I and FN gene expression by enhancing their transcription. It was hypothesized that the AcAld-mediated effect was due to the formation of adducts with proteins (11). This hypothesis was supported by using two inhibitors of the AcAld-protein adduct formation: PLP and PHMB. AcAld may condense with amino groups of several proteins (Schiff's bases) and form AcAld-protein adducts (31) that are stabilized in vitro by reducing equivalents (32). AcAld may also react with thiol groups of proteins and thiol compounds such as cysteine and glutathione (18). PLP is known to bind to free amino groups and, therefore, can interfere with the AcAld condensation with proteins. PHMB can modify the protein thiol groups and impair the AcAld binding to these groups in proteins (18). Nomura and Lieber (18) showed that PHMB only blocks 28% and PLP 51% of AcAld-protein binding. In contrast, there is experimental evidence that these compounds strongly reduced TCA-precipitable radioactivity ([^{14}C]AcAld) in the FSC layers, suggesting that the AcAld-protein adducts occurred intracellularly and/or in the cell membrane. This confirms previous reports that showed AcAld adduct formation with cell proteins (18,31) and the presence of adducts in human liver cells after chronic alcohol consumption (33). The disparity in the percentage of AcAld binding inhibition was higher in our experiments than in those of Nomura and Lieber and might be explained by the different experimental models. Nomura and Lieber used hepatic microsomes, whereas we described the effect of PHMB and PLP on the entire cell where possible sites of AcAld-protein binding and of inhibition are more pronounced. When PLP or PHMB was added to FSC cultures a significant reduction of AcAld-protein adducts was observed in the cell layer (Table 2) and a complete inhibition of the stimulatory effect of AcAld was obtained on Coll I and FN production (Fig. 2). This inhibitory activity occurred at the level of gene regulation.

AcAld is metabolized in cells by aldehyde dehydrogenase. The metabolization of AcAld by FSC was demonstrated (i) by a higher rate AcAld disappearance in culture media with cells than in media without (Fig. 1) and (ii) by the AcAld concentrations, which dropped over time, but remained high when the cells were cultured with cyanamide, an inhibitor of aldehyde dehydrogenase (11). The AcAld concentrations in the cultures were either the same or higher (see Fig. 1) than the hepatic venous blood levels observed in baboons given alcohol (34). However, it is known that AcAld concentrations are higher in the liver than in the blood (35) and, therefore, concentrations used in cell culture media can be expected to be close to intracellular hepa-

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tic AcAld levels. AcAld metabolism may lead to a change in the redox state of the cell by increasing the NADH/NAD ratio (36). Lactate also increases the intracellular NADH/NAD ratio. Thus, the lactate results support previous findings that the effect of AcAld on the extracellular matrix is not mediated by changes in the redox state of cells (11) thus supporting the 'AcAld-adduct theory'. The molecular basis of the effects of Ac.ld-protein adducts on collagen synthesis are not yet completely understood. Changes in the immune response (37) and the development of antibodies, against either circulating (38) or liver (33) AcAld-modified epitopes in humans, have been described as possible mechanisms of the alcohol-induced liver injury. However, in this study a 'transcriptional' effect of the AcAld-protein adduct is described on the Coll I and FN genes. In our opinion two hypotheses can be made. Fir. :. AcAld can form adducts with histone H1 and thus impair its DNA binding properties and lead to an inability to repress genes in the liver (39). Second, AcAld might condensate with cell-associated (intracellular and membrane-bound) type I procollagen molecules and therefore impair the feedback effect of collagen on its synthesis (40).

In conclusion, ethanol may act on FSCs as a fibrogen- factor in the liver through its immediate metabolite, acetaldehyde, whereas lactate does not seem to play a role in ethanol-induced liver fibrosis. Moreover, AcAld might stimulate procollagen I and FN gene transcription in FSCs through the formation of adducts with proteins.

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